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Neonicotinoid insecticides inhibit cholinergic neurotransmission in a molluscan (*Lymnaea stagnalis*) nervous system

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ABSTRACT

Neonicotinoids are highly potent and selective systemic insecticides, but their widespread use also has a growing impact on non-target animals and contaminates the environment, including surface waters. We tested the neonicotinoid insecticides commercially available in Hungary (acetamiprid, *Mospilan*; imidacloprid, *Kohinor*; thiamethoxam, *Actara*; clothianidin, *Apacs*; thiacloprid, *Calypso*) on cholinergic synapses that exist between the VD4 and RPeD1 neurons in the central nervous system of the pond snail *Lymnaea stagnalis*. In the concentration range used (0.01–1 mg/ml), neither chemical acted as an acetylcholine (ACh) agonist; instead, both displayed antagonist activity, inhibiting the cholinergic excitatory components of the VD4-RPeD1 connection. Thiacloprid (0.01 mg/ml) blocked almost 90% of excitatory postsynaptic potentials (EPSPs), while the less effective thiamethoxam (0.1 mg/ml) reduced the synaptic responses by about 15%. The ACh-evoked membrane responses of the RPeD1 neuron were similarly inhibited by the neonicotinoids, confirming that the same ACh receptor (AChR) target was involved. We conclude that neonicotinoids act on nicotinergic acetylcholine receptors (nAChRs) in the snail CNS. This has been established previously in the insect CNS; however, our data indicate differences in the background mechanism or the nAChR binding site in the snail.

Here, we provide the first results concerning neonicotinoid-related toxic effects on the neuronal connections in the molluscan nervous system. Aquatic animals, including molluscs, are at direct risk while facing contaminated surface waters, and snails may provide a suitable model for further studies of the behavioral/neuronal consequences of intoxication by neonicotinoids.

1. Introduction

Neonicotinoids are the newest generation of highly potent and selective systemic insecticides used as agrochemicals or to protect plants in the household from sucking insects (Tomizawa and Casida, 2005). Imidacloprid was the first neonicotinoid introduced to the market in the 1990s, followed by its homologues thiacloprid, thiamethoxam, nitenpyram, acetamiprid, clothianidin and dinotefuran. During the next 20 years neonicotinoids successfully replaced the carbamates and organophosphates as soil or seed treatments (Jeschke et al., 2011).

All neonicotinoid molecules are structurally related to nicotine, a natural alkaloid insecticide, but the positively charged nitrogen atom is replaced by other moieties, resulting in the nitro-substituted imidacloprid and thiamethoxam or the cyano-substituted acetamiprid and thiacloprid. The metabolites of some of these neonicotinoids also possess bioactivity; for example clothianidin, the active metabolite of thiamethoxam, has an even stronger effect in the insect CNS than thiamethoxam itself (Benzidane et al., 2010). The toxic effect of the neonicotinoids is based on their strong agonist binding to nicotinic acetylcholine receptors (nAChRs), which is confined to the CNS in the insect. While the binding is largely irreversible, it competes with natural acetylcholine (ACh) binding at the same receptors (Tomizawa and Casida, 2003). The selective effect of neonicotinoids on insects mainly results from differences between insect and mammalian nAChRs, but is also due to a structural feature of neonicotinoids, namely a pharmacophore which lacks a charged nitrogen and enables the molecule to more easily cross the brain–blood barrier in the insect nervous system (Tomizawa, 2013; Liu et al., 2010).

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The widespread use of neonicotinoid type insecticides also triggers environmental concerns that spread beyond the exposed areas and the target organisms (insects). When used as a seed coating, the water solubility and systemic action of imidacloprid, thiamethoxam or clothianidin allow these chemicals to travel from the seedlings to other parts in the growing plant, causing them to appear in the guttation droplets, pollen or even honey made from the treated plants (Girolami et al., 2009; Chen et al., 2014). Neonicotinoids, therefore, also pose a potential risk for non-target pollinator insects and other organisms that come into contact with the treated plants. It is possible that the recent appearance of colony collapse disorder (CCD), resulting in a seriously decreased number of bees worldwide, can be linked to the intensive use of globally distributed neonicotinoid insecticides in agricultural areas (Gill et al., 2012; Cressey, 2013; Dicks, 2013; van der Sluijs et al., 2013; Székács et al., 2015). Recent data demonstrate that neonicotinoid chemicals and their metabolites persist and accumulate in soil (Goulson, 2013) and also appear in aquatic ecosystems, potentially affecting a number of invertebrate taxa initially considered as non-target organisms (Jeschke et al., 2011; Morrissey et al., 2015). Most recent studies suggest a declining abundance of macro-invertebrates (Van Dijk et al., 2013) and a shift of species composition, in particular in aquatic communities where neonicotinoid pesticides are present in the environment (Liess and Von Der Ohe, 2005; Beketov et al., 2013).

Acute and chronic toxicity assessment studies of neonicotinoids most often use aquatic arthropods (crustaceans and insects), which provide well established and budget sensitive models for toxicological testing (Jemec et al., 2007; Daam et al., 2013; Pisa et al., 2015). Toxicological bioassays usually give informations regarding particular endpoints while physiological experiments may reveal target mechanisms and also give tools for comparative studies. Direct physiological/pharmacological analysis of the cellular/neuronal changes behind the neuronal alterations, however, often requires a far more complex and sophisticated system (Matsuda et al., 2001; Deglise et al., 2002; Palmer et al., 2013).

In the isolated central nervous system (CNS) of selected gastropods (i.e. the pond snail, *Lymnaea stagnalis* or the edible snail, *Helix pomatia*) the identifiable giant neurons (with diameters up to 100 μm) allow potential toxic effects to be examined using intracellular electrophysiology techniques. Acetylcholine is a neurotransmitter and modulatory substance both in the CNS and the periphery of these organisms; moreover, cholinergic receptor subtypes including nAChRs have also been established (Walker et al., 1996; Vulfius et al., 2005; van Nierop et al., 2006; Krajcs et al., 2014). Therefore the identifiable snail neurons provide a suitable tool to characterize the interactions between the ACh receptors (AChRs) and potentially toxic substances including heavy metals, insecticides or mycotoxins (Arvanov et al., 1993; Gyori et al., 1994, 2007). The roles of many identified neurons in controlling the relatively simple behavioral patterns of the animals have also been established (Chase, 2002), meaning that toxin-evoked functional changes of the nervous system will refer to known behavioural alterations of the intact animal (Dobranskyte et al., 2004; Vehovszky et al., 2007; Das and Khangarot, 2011).

The visceral VD4 neuron in the CNS of *L. stagnalis* provides monosynaptically transmitted inputs to a number of its followers including the symmetrically located pair of giant neurons (LPeD1 and RPeD1) of the pedal ganglia (Syed and Winlow, 1991). Both connections have also been shown to re-form between the isolated neurons when placed in culture conditions (Syed et al., 1990; Hamakawa et al., 1999). The first, excitatory component of these monosynaptic connections provides a suitable in vitro model while studying synaptogenesis (Feng et al., 1997; Woodin et al., 2002), or toxin-induced alterations of cholinergic neurotransmission (Woodall et al., 2003; Onizuka et al., 2012).

We tested the effects of commercially available insecticides that contain neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam) on the identified cholinergic synapses between VD4 and RPeD1 neurons in the isolated CNS. Our results confirm that neonicotinoid insecticides act on the AChRs in the molluscan CNS, and also demonstrate differences in sensitivity and kinetics between the AChRs of different locations (synaptic and extrasynaptic) on the same neuron.

This study provides the first data on the effects of neonicotinoids on molluscs, an example of non-target members of the aquatic ecosystem exposed to the harmful side effects of intensive pesticide use.

2. Materials and methods

2.1. Animals

Adult specimens of the pond snail *L. stagnalis* were collected in the Balaton area (Hungary), kept in tanks filled with filtered Balaton water and fed on lettuce ad libitum.

2.2. Chemicals

The individual insecticides tested were used in the form of the commercially available products in Hungary, namely acetamiprid (*Mospilan*, Sumi Agro), imidacloprid (*Kohinor*, Makteshim Agan), thiamethoxam (*Actara*, Syngenta), clothianidin (*Apacs*, Arysta Life Science) and thiacloprid (*Calypso*, Bayer). Other chemicals were obtained from Sigma–Aldrich Chemie GmbH, Germany. All the chemicals were dissolved in *Lymnaea* saline immediately prior to the experiments. The accurate concentrations of the active ingredients in each neonicotinoid product were confirmed by GC/MS chromatography.

Electrophysiological experiments used physiological *Lymnaea* solution (normal saline) made from distilled water and containing NaCl (51.5 mM), KCl (1.7 mM), $CaCl_2$ (4.1 mM), $MgCl_2$ (1.5 mM), buffered with Hepes (5 mM) and set to pH 7.9. In some experiments a modified (HiDi) saline was used, with elevated amounts of the following divalent cations: $CaCl_2$ (24.6 mM) and $MgCl_2$ (5 mM).

2.3. Electrophysiological recording

The electrophysiological tests were carried out on isolated *Lymnaea* CNS preparations placed in a perfusion chamber filled with normal saline. The upper layer of the connective tissue covering the dorsal surface of the suboesophageal ganglion was removed mechanically first, and then the inner layer was digested with 0.1% protease treatment (Sigma type XIV) for 5 min before removing.

Both the pedal RPeD1 and the visceral VD4 giant neurons were visually identified by their size, position and colour (Syed and Winlow, 1991) before penetration by microelectrodes for electrophysiological recording. The RPeD1 neuron was impaled by two independent microelectrodes to inject current into the cell body for membrane polarization while simultaneously recording synaptically-evoked potentials or ACh-induced membrane responses from the same neuron. Controlled amounts of ACh were applied ionophoretically onto the cell surface of the RPeD1 neuron by placing a low resistance micropipette filled with 100 mM ACh in distilled water adjacent to the cell and passing positive current pulses (1 s duration, 10–50 nA amplitudes). Spontaneous leakage of ACh from the pipette was prevented by applying a constant retaining current of −0.5 nA between the application pulses. Both the recording electrodes and the injecting pipette were made from 1.2–1.4 mm diameter filamented borosilicate glass tubes (Harward Apparatus Ltd.), pulled to a tip resistance of 6–10 MΩ.